# Macrophage targeting in cancer

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REVIEW

# Macrophage targeting in cancer

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**Tumorigenesis is not only determined by the intrinsic properties of cancer cells but also by their interactions with components of the tumor microenvironment (TME). Tumor-associated macrophages (TAMs) are among the most abundant immune cells in the TME. During initial stages of tumor development, macrophages can either directly promote antitumor responses by killing tumor cells or indirectly recruit and activate other immune cells. As genetic changes occur within the tumor or T helper 2 ($T_H2$) cells begin to dominate the TME, TAMs begin to exhibit an immunosuppressive protumor phenotype that promotes tumor progression, metastasis, and resistance to therapy. Thus, targeting TAMs has emerged as a strategy for cancer therapy. To date, TAM targeting strategies have focused on macrophage depletion and inhibition of their recruitment into the TME. However, these strategies have shown limited therapeutic efficacy, although trials are still underway with combination therapies. The fact that macrophages have the potential for antitumor activity has moved the TAM targeting field toward the development of TAM-reprogramming strategies to support this antitumor immune response. Here, we discuss the various roles of TAMs in cancer therapy and their immunosuppressive properties, as well as implications for emerging checkpoint inhibitor–based immunotherapies. We review state-of-the-art TAM-targeting strategies, focusing on current ones at the preclinical and clinical trial stages that aim to reprogram TAMs as an oncological therapy.**

**Keywords:** macrophage; TAM; cancer; targeting; reprogramming; tumor microenvironment

## Introduction

Tumor-associated macrophages (TAMs) are among the most abundant immune cells in the tumor microenvironment (TME).[1–3] Originally, they were thought to be antitumoral, owing to their ability to kill tumor cells *in vitro*.[4–6] Indeed, at the earliest stages of tumor onset, the immune system may promote activation of T cells and macrophages to clear tumor cells.[7] However, once a tumor progresses past an initial stage, the TME is influenced by cancer cells to provide support for their growth, and even though there may still be antitumor macrophages present, the majority of macrophages are "educated" to enhance tumor progression and metastasis (Fig. 1).

High infiltration of TAMs (as demonstrated by an accumulation of macrophage-related growth factors/chemokines/cytokines,[8–12] and/or large numbers of macrophages in tumors[1,9,13–18]) correlates with poor prognosis and reduced patient survival in several different types of cancer, including human breast, gastric, oral, ovarian, bladder, and thyroid cancers, non-small cell lung carcinoma (NSCLC), and Hodgkin's lymphoma.[19–21] Consistent with those correlative data, *in vivo* experiments in mouse models of cancer have indicated that TAMs are protumoral. Originally, these conclusions were derived from genetic ablation of *Csf1* (which expresses macrophage colony–stimulating factor 1 required for macrophage survival and maturation) and, thereby, elimination of macrophages in a mammary carcinoma mouse model, which resulted in delayed tumor development and reduced pulmonary metastasis.[22] Reciprocally, transgenic expression of *Csf1* in wild-type and *Csf1*-null mice accelerated tumor invasive behavior, leading to pulmonary metastasis increase.[22] Similar data have been obtained in many other mouse models

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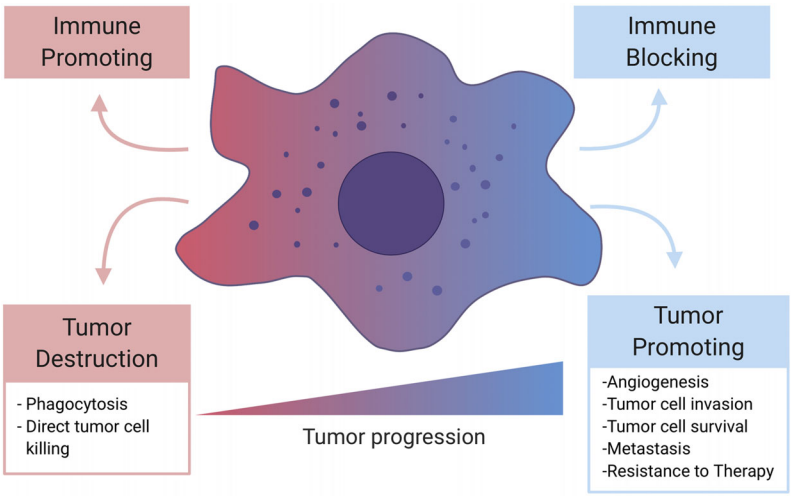


**Figure 1.** **Macrophages can promote an antitumor response, but these responses are abrogated as As T helper 2 ($T_H2$) cells start dominating the TME. Macrophages can promote an antitumor response (red boxes) by directly phagocytosing and/or killing tumor cells, or indirectly by recruiting and/or presenting tumor antigens to activate other immune cells. These antitumor responses, however, are abrogated as $T_H2$ cells start dominating the TME. TAMs then exhibit an immunosuppressive phenotype (blue boxes) that resembles that of macrophages involved in tissue development and repair, thereby aiding in tumor progression and metastasis.**

of cancer using similar or different strategies for macrophage ablation.[2,23,24] Together, the data suggest that TAMs, in both mouse and human contexts, promote tumor progression to metastasis (Fig. 2).

## TAMs ontogeny in the TME: breaking the dogma

The origin of tissue-resident macrophages (TRMs) in adults was thought to be restricted to the mononuclear phagocytic system, whereby hematopoietic stem cells differentiate to common myeloid progenitors, that in turn differentiate to granulocyte-monocyte progenitors, that give rise to monocyte-dendritic progenitors, that differentiate to monocytes and then into macrophages.[25] However, this view has been shown to be inadequate, as several groups using lineage tracing methods have shown that TRMs can be derived from three developmentally distinct sources: embryonic precursors from the yolk sac, embryonic precursors from fetal liver (after seeding from the yolk sac), and postnatally from monocytes derived by hematopoiesis primarily in the bone marrow (BM).[26–31] Consistent with these diverse origins in normal development, TAMs—considered to be exclusively derived from monocytes that infiltrate a tumor—have also been shown to originate from yolk sac–derived TRMs. For example, in pancreatic ductal adenocarcinoma (PDAC) in mice, both inflammatory monocytes and TRMs are TAM sources.[32] Zhu *et al.* demonstrated that TAMs of embryonic origin were able to proliferate *in situ* during tumor progression, and that they are transcriptionally different from TAMs derived from monocytes. In the lung, embryonically derived tissue-resident interstitial macrophages largely contribute to the pool of TAMs, and their accumulation associates with tumor growth. $CCR2^+$-dependent monocytes also contribute to the TAM pool, but they seem to be associated with tumor spread rather than growth.[33] By contrast, in lung metastases models, metastasis-associated macrophages (MAMs) are exclusively derived from monocytes.[34,35] Furthermore, in MMTV-PyMT tumors (a mouse mammary tumor model), TAM origin is exclusively from monocytes.[36,37]

Data regarding the origin of TAMs in the brain are conflicting; some groups found that resident microglia are the main source of glioma-associated macrophages,[38] while others claim that monocytes are the main source.[39] It has been highlighted that there are technological caveats with the models to study the origin of glioma TAMs; for example, experiments that have used irradiation might promote bias because irradiation causes the disruption of the blood–brain barrier, which could result in an increased accumulation of monocyte-derived


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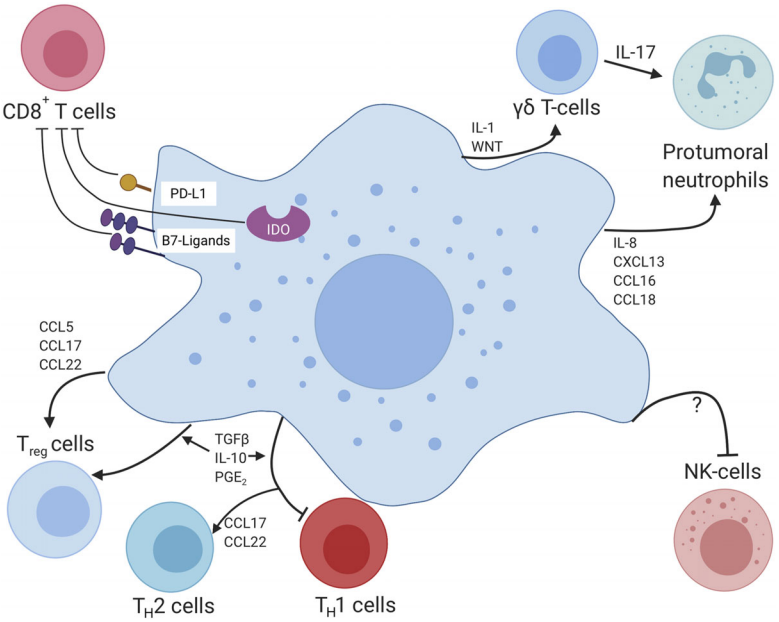


**Figure 2.** **Protumor macrophages are immunosuppressive. Immunosuppressive TAMs express immune checkpoint ligands, such as programmed-death ligand 1 (PDL1) and B7 ligands, which directly inhibit cytotoxic T cell functions. T cell cytotoxicity can also be directly inhibited by macrophage-mediated tryptophan depletion (macrophage indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme in the degradation pathway of tryptophan). Protumor macrophages secrete prorestorative cytokines TGF-β (transforming growth factor beta), interleukin (IL)-10, and $PGE_2$ (prostaglandin $E_2$), which leads to downregulation of MHC class II (major histocompatibility complex II), and thus, a diminished $T_H1$ differentiation and in the expansion of $T_{reg}$ cells (regulatory T cells). TAMs also promote immune suppression through the recruitment of $T_H2$ cells and $T_{reg}$ cells by the production of chemokines, such as chemokine ligand (CCL) 17 and CCL22. Protumor neutrophils are recruited by IL-8, CXCL13 (C-X-C motif chemokine ligand 13), CCL16, and CCL18. γδ T cells accumulate in the TME via macrophage secreted IL-1 and WNT pathway–associated molecules,[235,236] and these cells recruit protumor neutrophils via IL-17.[237,238]**

TAMs.[40] A recent publication on single cell sequencing of human gliomas identified two TAM subsets: one that presumably has an embryonic tissue–resident origin, as the gene expression profile showed an enrichment of microglial genes, and a second subset that indicates an adult BM–derived monocyte origin.[41] Adult monocyte–derived TAMs have altered metabolism, upregulated immunosuppressive cytokines, and a gene signature that correlates with poor survival in low-grade glioma. These findings suggest that while monocyte-derived TAMs significantly infiltrate the tumor, they do not entirely adopt the phenotype of microglial-derived TAMs.

It is now becoming evident that TAMs from different developmental origins and pathways have both overlapping and distinct functions within the same tumor. However, the origin of many of the TAM populations assessed in human patients, and even in some mouse studies, is not known. What is relatively clear, however, is that elucidating TAM ontogeny will help to inform therapeutic approaches. For example, a recent publication[42] using single-cell sequencing suggested that two distinct TAM subsets in colorectal cancer arise from either monocytes or TRMs and have different gene signatures. The equivalent murine TAM subsets were shown to have differential sensitivity to CSF1R blockade; anti-CSF1R treatment mainly depleted the TAM subset that emerged solely from monocytes, sparing TAMs with an angiogenic-related signature that most likely emerged from TRMs.[42]

## Direct protumor activities of TAMs

In this section, we give a brief overview of protumor functions of TAMs (which have also been covered in several excellent reviews[2,23,43–46]). TAMs are involved in angiogenesis, which is an important part of a tumor's ability to expand and metastasize. Depletion of TAMs by abrogating *Csf1* expression

in a mammary cancer mouse model blocked the "angiogenic switch," a characteristic of the transition from benign to malignant tumors,[47] while depletion of Tie2$^+$ TAMs inhibited glioma neovascularization in the mouse brain.[48,49] Genetic restoration of the macrophage populations in the tumors from both models rescued the blood vessel phenotype.

Hypoxia is a major driver of angiogenesis, and hypoxic areas attract TAMs by the release of hypoxia-induced chemoattractant molecules, such as C-X-C motif chemokine ligand (CXCL) 4, chemokine ligand (CCL)-2, vascular endothelial growth factor (VEGF), and others.[50] TAMs respond to hypoxia by upregulating hypoxia-inducible transcription factors and their downstream targets, which include a wide range of proangiogenic factors, such as transforming growth factor-beta (TGF-β) and others.[50–54]

Macrophages accumulate at the invasive front of tumors during malignant transformation[22,55]; these macrophages directly help tumor cells escape from primary tumor sites into blood or lymphatic vessels. TAMs aid in the epithelial–mesenchymal transition (EMT) of tumor cells in which tumor cells lose cell–cell junctions and acquire a motile mesenchymal phenotype.[56] TAMs contribute to the EMT process by secreting soluble factors, such as interleukin (IL)-1β, IL-18, tumor necrosis factor alpha (TNF)-α, TGF-β, and others,[57–59] as well as extracellular matrix (ECM)–degrading proteins, such as cathepsins, metalloproteinases (MMP7, MMP2, and MMP9), and serine proteases that allow migration of the tumor cells.[60] Other macrophage-mediated tumor invasion–related factors include osteonectin, which plays a role in collagen fiber deposition and expression of MMPs,[61] and Wnt5a, which stimulates cancer cell motility.[62] Also secreted by TAMs, the protein SPARC (secreted protein acidic and rich in cysteine) is required for the migration of tumor cells, as its genetic ablation leads to a decrease in metastasis.[63,64] SPARC supports fibronectin and vitronectin interactions with tumor cell–expressed integrins, which leads to a traction force along ECM fibers that allows tumor cells to travel through the stroma toward the vasculature.[64,65]

A tripartite arrangement of Tie2$^+$ TAMs, tumor cells, and endothelial cells, the TME of metastasis (TMEM) is involved in tumor cell intravasation—the presence of the TMEM, for example, is a predictor of poor prognosis in breast cancer.[66] Processes in the TMEM are in some cases mediated by a paracrine loop that involves the production and secretion of EGF family ligands by macrophages to promote tumor cell migration, and the production of CSF1 by tumor cells to promote macrophage recruitment and survival.[55,67] Furthermore, Tie2$^+$ TAMs in the TMEM express and secrete vascular endothelial growth factor A (VEGFA), which causes local loss of vascular junctions and transient vascular permeability that allows for escape of tumor cells.[68] Once tumor cells have escaped into the blood or lymphatic circulation, they evade immune system recognition and attack, thereby gaining the means to survive and proliferate in new environments.[69] Macrophages and/or their progenitors recruited from the BM are key players in the formation of premetastatic niches, as they help tumor cells evade immune cell recognition and they aid in the preparation of distant sites for tumor cells to colonize.[69,70] Pre-metastatic niches are created by systemic influences of primary tumors acting in part through the BM and, locally, via ECM formation that results in the recruitment of myeloid cells, including monocytes, which then differentiate *in situ*; they are attracted by secreted factors CCL2, CSF1, VEGF, TNF-α, TGF-β, and tissue inhibitor of metallopeptidase-1, as well as by exosomes.[70–73] Myeloid cells in the premetastatic niche attract tumor cells by secreting chemokines and then remodeling the ECM (via MMPs, integrins, and lysyl oxidase) to promote angiogenesis, EMT, and extravasation. This enhances both tumor cell tropism and their abilities to seed and survive.[72] Once tumor cells arrive at these metastatic sites, a distinct population of macrophages known as MAMs promotes the extravasation of tumor cells and their persistent growth. Depletion of this distinct population leads to a decrease in the tumor cell extravasation rate and, thereby, a failure of establishment of new metastases,[74] which leads, in some cases, to improved survival of animals.[34,74–76] MAMs are recruited by CCL2:[34] activation of the CCL2–CCR2 axis triggers a chemokine cascade (including CCL3) that results in maturation of MAMs and adhesion via $\alpha_4$ integrin to vascular cell adhesion molecule 1 (VCAM-1) expressed on the surface of tumor cells. Adhesion activates PI3K–Akt survival signaling[77,78] that facilitates lung metastatic

seeding by breast cancer cells.[75] In prostate cancer, bone metastases triggering the CCL2–CCR2 axis promotes the activation of osteoclasts, which leads to enhanced bone resorption and, in turn, the liberation of entrapped growth factors that stimulate the generation of other bone metastases.[79] Furthermore, as in lung metastases, MAM binding to VCAM-1 delivers a survival signal that leads to the protection of cancer cells from proapoptotic cytokines, such as TNF-related apoptosis-inducing ligand.[77] In addition to the protumor functions of TAMs or MAMs that directly enhance cancer malignancy, other TAM/MAM activities can regulate other immune cells, such as T cells, to attenuate their otherwise antitumor activity (Fig. 2).[35,76] Furthermore, TAMs/MAMs can limit the effectiveness of classical cancer therapies, such as chemo- and radiotherapy, as well as biological therapies, including immuno-oncological therapies (Fig. 1). These data suggest that targeting TAMs and/or MAMs would be an effective strategy in cancer therapy.

In the remainder of our review, we concentrate on the roles of TAMs in anticancer therapies, both classical and immunological. We will discuss TAM immunosuppressive properties and the implications of this on emerging checkpoint inhibitor–based immunotherapies. Importantly, we review the state-of-the-art of TAM-targeting strategies, focusing on current strategies at the preclinical and clinical trial stages that aim to reprogram/reeducate TAMs as an effective oncological therapy.

## TAMs and therapy

### *TAMs impair cancer standard therapy response*

TAMs are able to mediate resistance to chemotherapy.[80] For example, inhibiting CSF1 activity can reverse chemoresistance of human breast cancer cell lines in xenograft mouse models.[81] Other work has shown that breast cancer murine models treated with paclitaxel and anti-CSF1 receptor signaling antagonists had reduced tumor burden and increased T cell infiltration when compared with models treated with paclitaxel only.[82] Furthermore, biopsies from cancer patients undergoing neoadjuvant therapy show higher numbers of macrophages in tumors compared with cancer patients who receive only surgery.[83]

Chemotherapeutic agents, such as 5-fluorouracil (5-FU) and doxorubicin, affect TAM phenotypes. In colorectal cancer, 5-FU promotes macrophage secretion of diamine putrescine, which prevents tumor cell apoptosis.[84] It also causes CCL22–PI3K–AKT signaling activation in TAMs that stimulates migration and invasion of tumor cells.[85] Doxorubicin treatment leads to the accumulation of perivascular TAMs that express VEGF and supports angiogenesis, leading to enhanced tumor invasion.[80] Inhibiting the recruitment of TAMs led to a reduced rate of tumor relapse after chemotherapy.

Radiotherapy aims to target cells that have compromised DNA repair mechanisms (e.g., tumor cells), but certainly it also affects normal cells in the microenvironment. It has been shown that irradiated macrophages remained viable and metabolically active, with a proinflammatory phenotype (as there is upregulation of proinflammatory macrophage markers CD80, CD86, and HLA-DR and downregulation of prorestorative macrophage markers CD163, MRC1, VCAM, and IL-10). However, these irradiated macrophages are still able to promote angiogenesis and tumor cell invasion.[86] Furthermore, during irradiation-induced wound repair, growth factors and chemokines, such as IL-1, TNF-α, and TGF-β, recruit macrophages with a tissue repair–associated phenotype; and this contributes to tumor recurrence.[87]

### *TAMs can enhance standard therapy responses*

In contrast to the above, TAMs can also contribute to the therapeutic efficacy of standard anticancer strategies.[88] The chemotherapeutic agent cyclophosphamide induces the secretion of CCL4, IL-8, VEGF, and TNF-α by treated tumor cells in a model of refractory B cell leukemia. These factors induce macrophage infiltration in the BM and increase phagocytic activity.[89] Even though a high density of TAMs is associated with poor prognosis and distant metastasis in PDAC, this association is lost in patients who have undergone postsurgical adjuvant chemotherapy.[90] This can be explained by both a decreased number of protumoral TAMs, as there were fewer $CD206^+$ and $IL\text{-}10^+$ TAMs at the tumor–stroma interface, and *in vitro* experiments showing that gemcitabine-treated macrophages show increased expression of cytotoxic activity–related genes, thus consistent with the macrophages becoming more tumoricidal.[90] In

colorectal cancer patients, high TAM abundance has been associated with better disease-free survival, but only when patients had undergone 5-FU adjuvant therapy.[91] *In vitro* experiments showed that there was a synergistic effect of macrophage presence and 5-FU treatment on colorectal cancer cell death.[91]

Regarding radiotherapy, low-dose γ-irradiation in various murine models causes normalization of aberrant vasculature, enhances the recruitment of T cells, and causes prolonged survival.[92] Low-dose γ-irradiation was also shown to promote the differentiation of monocytes and macrophages toward an $iNOS^+$ antitumor macrophage phenotype, and with expression of T cell–attracting chemokines and suppression of angiogenic and immunosuppressive factors. In this model, therefore, macrophage activation promoted T cell recruitment and cytotoxicity.[92]

### *TAMs are immunosuppressive*

Revolutionizing the field of oncology, immunotherapeutic approaches increase cancer survival by stimulating the tumoricidal abilities of cytotoxic lymphocytes. Immune checkpoint inhibitor–based therapies targeting programmed death 1 (PD1), programmed death ligand 1 (PDL1), and cytotoxic T lymphocyte antigen 4 (CTLA-4) axes can reinvigorate T cell recognition and cytotoxicity against tumor cells. However, their effectiveness relies on the presence of baseline patient immune responses that are "unleashed" after immunotherapy to kill cancer targets, a concept recently summarized by a "hot" and "cold" tumor paradigm.[2,93,94] Hot tumors are characterized by high infiltration of cytotoxic T cells that are anergic; T cell checkpoint inhibition therapies are most effective against these tumors. Cold tumors are characterized by the absence of T cells at the tumor bed and edges; they are the most challenging to eradicate and are associated with poor prognosis, as T cell priming fails.[94] Cold tumors, moreover, are characterized by low mutational burden, poor antigen presentation, and tumor-intrinsic insensitivity to T cell–mediated killing.[95]

Currently, it is estimated that only 20–40% of cancer patients respond to immunotherapy.[96] The mechanisms behind immunotherapy resistance can be classified as *tumor cell–intrinsic*, which includes absence of tumor cell antigenic proteins, and insensibility to T cells, and *tumor cell–extrinsic*, which includes the absence of T cells, the presence of additional inhibitory immune checkpoints, and/or the presence of immunosuppressive cells, such as TAMs (reviewed in Refs. 2, 97, and 98).

The presence/absence of T cells in conjunction with the presence of immunosuppressive cells has led to further subdivision of the hot and cold tumor dichotomy.[95] Tumors defined as *altered–excluded* are characterized by no T cell infiltrate inside the tumor bed but an accumulation of the cells at the invasive margin; although there is an intrinsic ability of the immune system to mount a T cell response, the tumor escapes because there is a physical barrier hindering T cell infiltration. This physical barrier can be TAMs, as they can promote *T cell trapping* at the border of the tumors by forming long-lasting interactions with $CD8^+$ T cells.[99,100]

Tumors defined as *altered–immunosuppressed* are characterized by poor, although not absent, T cell infiltration due to the presence of an immunosuppressive environment (e.g., myeloid-derived suppressor cells (MDSCs),[101] TAMs, and/or regulatory T cells) within the tumor. Preclinical studies have demonstrated that resistance to immune checkpoint therapy can be circumvented by targeting macrophages with CSF1R inhibitors in colorectal cancer.[102] Furthermore, a combination of anti-PD1 and anti–CSF1R was shown to induce the regression of transplant $BRAF^{V600E}$–driven mouse melanomas.[103] Depletion of TAMs restored T cell migration and infiltration and improved the efficacy of anti–PD1 immunotherapy in lung squamous cell carcinoma altered–excluded tumors.[99]

**TAM-mediated immunosuppression mechanisms.** In therapeutic situations, TAMs can promote an antitumor response by directly phagocytosing and/or killing tumor cells or, indirectly, by recruiting and/or presenting tumor antigens to activate cytotoxic T cells and natural killer (NK) cells (Fig. 3). For example, treatment of murine cancer models with CCL16, Toll-like receptor (TLR) 9 ligand CpG, and anti-IL-10 receptor antibodies led to an accumulation of macrophages at the site of the tumor, as well as macrophage cytokine secretion. This, in turn, caused immune system activation and impairment of tumor growth and metastasis.[104] Treatment of murine breast cancer models with granulocyte-macrophage colony-stimulating factor (GM-CSF)

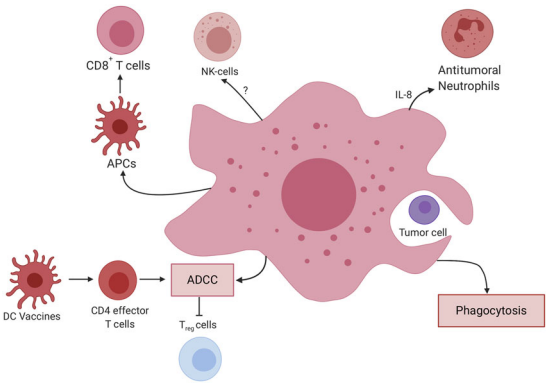


**Figure 3.** **Macrophages exert antitumor functions by promoting immune activation. Macrophages can promote an antitumor response by directly phagocytosing and/or killing tumor cells by ADCC. They can promote antitumor response indirectly by recruiting activated immune cells, such as antitumor neutrophils via IL-8, NK cells, and $CD8^+$ T cells and/or by presenting tumor antigens to activate cytotoxic T cells. Macrophages can also indirectly inhibit $T_{reg}$ cell accumulation in the TME.**

also led to an impairment of tumor growth and metastasis by blocking macrophage VEGF activity and reducing protumoral macrophage cytokines IL-10 and IL-4.[105] However, these antitumor responses are abrogated in the T helper type 2 ($T_H2$) cells dominating the TME.[1,23] Under these conditions, TAMs exhibit an immunosuppressive phenotype that resembles the phenotype of macrophages involved in tissue development and repair.[97,106]

Immunosuppressive TAMs are characterized by a secretory profile consisting of low levels of inflammatory cytokines, such as IL-18, IL-12, TNF-α, and interferon gamma (IFN-γ), and high levels of anti-inflammatory/prorestorative cytokines, such as IL-10 and TGF-β.[107] TAM and tumor cell–derived prorestorative cytokines TGF-β, IL-10, and $PGE_2$ downregulate major histocompatibility complex (MHC) class-II molecules in TAMs. This results in diminished $T_H1$ differentiation (which in turn, results in decreased antitumor activity) and expansion of regulatory T ($T_{reg}$) cells, which are key players in tumor progression.[18] TAMs also promote immune suppression through the recruitment of $T_H2$ and $T_{reg}$ cells by the production of chemokines, such as CCL17 and CCL22,[108] and through the recruitment of eosinophils and naive T cells by the secretion of CXCL13, CCL16, and CCL18.[108–112]

TAMs express immune checkpoint ligands, such as PDL1, PDL2, B7-1 (also known as CD80), and B7-2 (also known as CD86), which directly inhibit cytotoxic T cell functions.[113–115] In a checkpoint inhibitor therapy setting, such TAM ligands compete with tumor cell ligands, reducing therapeutic efficacy. T cell cytotoxicity can also directly be inhibited by macrophage-mediated depletion of L-arginine (which is needed for the re-expression of the T cell receptor (TCR) after T cells have engaged with an antigen)[97] and by macrophage-mediated tryptophan depletion (macrophages express indoleamine 2,3-dioxygenase (IDO), a rate-limiting catabolic enzyme in the degradation pathway of tryptophan).[115] Furthermore, catalytic break down products of tryptophan also exert immunosuppressive roles.[116,117]

In metastatic sites, classical monocytes differentiate into a distinct, transient myeloid cell population (metastasis-associated macrophage precursors, or MAMPCs) that expresses mature macrophage markers and is able to suppress the cytotoxic activity of $CD8^+$ T cells through a reactive oxygen species–mediated mechanism.[35] MAMPCs are formally equivalent to a much-discussed but rarely adequately defined population of monocytic MDSCs,[101] suggesting their origin in many cancer types. Lineage tracking shows that the MAMPCs differentiate into MAMs that also directly suppress $CD8^+$ T cell killing, but in this case via expression of the CTLA4 ligands CD80 and CD86.[35]

The immunosuppressive properties of TAMs and their role in impairing immunotherapeutic responses (Fig. 2) highlight the benefit of removing them from the TME to improve cancer therapy. However, Hoves *et al.*[118] recently showed that activation of antitumor responses by macrophages using CD40 agonists was sufficient to create a proinflammatory environment that supported tumor responses of T cells that were otherwise resistant to checkpoint inhibitors. Considering that the CD40 agonist reprogramming of macrophages was time limited, Hoves *et al.* reported that costimulation with inhibitory anti-CSF1R and CD40 agonist more effectively induced T cell activation because anti-CSF1R led to depletion of the "re-programmed macrophages" before the tumor could re-educate them back to suppressive TAMs. This strategy was very effective in colorectal and mammary mouse tumors preclinical models.[118]

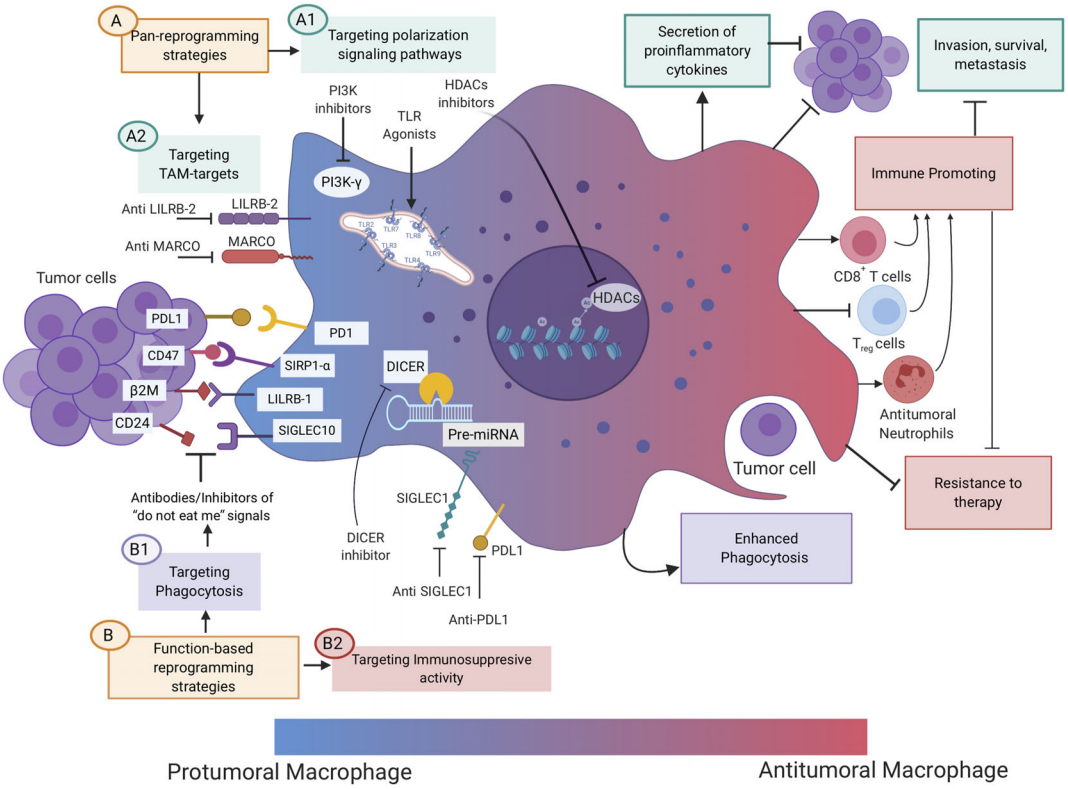


**Figure 4.** **Therapeutic strategies aimed at changing the phenotype of TAMs from a protumoral to an antitumoral state (macrophage reprogramming). TAM reprogramming strategies fall into two main categories: pan-reprogramming (A) and function-based reprogramming of TAMs (B). Pan-reprogramming includes strategies that target macrophage polarization signaling pathways (A1), such as using PI3Kγ inhibitors, TLR agonists, and HDAC inhibitors; and TAM-preferentially expressed targets (A2), such as MARCO and LILRB2. Function-based reprogramming of TAMs (B) includes targeting specific TAM functions, such as phagocytosis (B1), by targeting the macrophage tumor cell "do not eat me" signals SIRP1α–CD47, LILRB1–β2M, SIGLEC10–CD24, and PD1–PDL1, and macrophage immunosuppressive activities (B2) by inhibiting macrophage SIGLEC1, PDL1, or abrogating micro-RNA activity through DICER inhibition. Macrophage reprogramming leads to the secretion of proinflammatory cytokines, an enhancement of phagocytic ability, and macrophage-mediated immune promotion. These activities inhibit tumor progression, metastasis, and/or resistance to therapy. HDACs, histone deacetylases; TLRs, Toll-like receptors; PI3Kγ, phosphoinositide 3-kinase-γ; LILRB2, leukocyte immunoglobulin-like receptor-B2; MARCO, macrophage receptor with collagenous structure; PD1, programmed cell death 1; PDL1, PD1 ligand 1; SIRP1α, signal regulatory protein-α; LILRB1, leukocyte immunoglobulin-like receptor-B1; β2M, β2-microglobulin; SIGLEC10, sialic acid–binding Ig-like lectin-10; SIGLEC1, sialic acid–binding Ig-like lectin-1; pre-miRNA, pre-micro-RNA; $T_{reg}$ cells, regulatory T cells.**

Because macrophages can promote an antitumor cytotoxic cell response (Fig. 3), current therapies may benefit more from strategies that aim to reprogram TAMs from a pro- to an antitumoral state, rather than from those that aim to deplete them. These strategies (Fig. 4) will be discussed in detail below.

## Macrophages as a therapeutic target

Previous reviews have extensively discussed strategies to deplete or inactivate monocytes/macrophages (by targeting the CSF1–CSF1R axis or using bisphosphonates), as well as strategies to inhibit the recruitment of monocytes/macrophages to tumor sites (targeting CCL2–CCR2 signaling).[1,2,119] The main disadvantage of most of these macrophage depletion/inactivation strategies, however, is the systemic, indiscriminate targeting of macrophages, which leaves immune responses compromised and at a disadvantage in fighting external insults. Also, anti-CSF1 therapy with antibodies or small molecules leads to several adverse effects.[120] With regard to efficacy, TAM depletion in preclinical models causes intratumor


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**Table 1. Therapeutic strategies aimed at reprogramming the phenotype of TAMs from an antitumoral to a protumoral state**

| | | |
|---|---|---|
| **Pan-reprogramming strategies** | Targeting polarization signalling pathways | • Histone deacetylases (HDACs)<br>• PI3K$\gamma$ inhibitors<br>• TLR agonists |
| | Targeting TAM-preferentially expressed targets | • LILRB2<br>• MARCO |
| **Function-based reprogramming** | Targeting phagocytic activity of macrophages | • CD47 and SIRP1$\alpha$ axis<br>• $\beta_2$-M of MHC-I and LILRB1 axis<br>• CD24-SIGLEC10<br>• PDL1 and PD1 axis |
| | Targeting the immunosuppressive activity of macrophages | • PDL1 and PD1 axis<br>• SIGLEC1 (CD169)<br>• MicroRNAs, miR-340-5p |
| | Macrophage engineering | • Anti-Her2 CAR-macrophages |

neutrophils to become highly suppressive,[69,121,122] which counteracts the therapeutic benefit of a macrophage depletion approach.[69,121,122]

The suggestion that changing the phenotype of TAMs from an anti- to a protumoral state might be a superior therapeutic approach has led the field toward developing and testing several reprogramming strategies. Here, we have classified these TAM reprogramming strategies into two main categories: pan-reprogramming and function-based reprogramming of TAMs, although these are not necessarily exclusive (Table 1). Under pan-reprogramming, we place strategies whose main aim is to switch/polarize TAMs from a pro- to an antitumoral phenotype by targeting either macrophage polarization signaling pathways or specific TAM preferentially–expressed targets/markers (Table 1 and Fig. 4). Function-based reprogramming of TAMs includes therapeutic strategies aimed at targeting specific TAM functions, such as phagocytosis and immunosuppression, as well as strategies aimed at macrophage engineering (Table 1 and Fig. 4).

### *Pan-reprogramming of macrophages*

**Targeting macrophage polarization signaling pathways.** *HDACs.* Histone deacetylases (HDACs) are epigenetic regulators of gene expression through their activity of removing acetyl groups on histones. HDACs regulate the expression of many cancer initiation- and progression-related genes. Anomalous expression of HDACs happens in various types of cancer; high expression correlates with poor prognosis and poor survival.[123] Hence, several HDAC inhibitors have been made to treat a variety of tumors and are in clinical trials.[123]

Recently, the selective class IIa HDAC inhibitor TMP195 was shown to influence monocyte and macrophage behavior. Upon treatment with TMP195, macrophages secreted lower levels of CCL2 and higher levels of CCL1.[124] In a later study, treatment of a macrophage-dependent, autochthonous mouse model of breast cancer with TMP195 caused a reduction in both tumor burden and pulmonary metastases via altering macrophage phenotype.[125] TMP195 treatment promoted the recruitment of monocytes and their differentiation to highly phagocytic antitumor macrophages. Moreover, when TMP195 was given in combination with chemotherapeutic agents (paclitaxel and carboplatin), and, alternatively, in combination with checkpoint inhibitor anti-PD1, there was an increased therapeutic efficacy. To date, there has been no report of TMP195 in clinical trials.

Epigenetic adjuvant therapy can also disrupt the premetastatic niche by targeting mainly myeloid cells. In a recent publication by Lu *et al.*,[126] the authors showed that after removal of primary lung, breast, and esophageal tumors, the administration of low-dose DNA methyltransferase and HDAC inhibitors, 5-azacytidine and entinostat, respectively, inhibited the recruitment of immunosuppressive myeloid populations (MDSCs; see above)[101] through downregulation of the

chemokine receptors CCR2 and CXCR2. As a result, mice that received the treatment showed longer disease-free survival compared with mice treated with chemotherapy.

*PI3Kγ inhibitors*. Phosphoinositide 3-kinase gamma (PI3Kγ) is a leukocyte-restricted member of the important class of lipid kinases involved in activating many downstream signaling molecules, particularly in response to growth factor signaling.[127] In macrophages, PI3Kγ acts as a molecular switch that increases immunosuppressive activity, while decreasing immunostimulatory activity. Macrophage reprogramming has been achieved by both pharmacologic inhibition of PI3Kγ and genetic deletion of *Pik3cg*.[128,129]

Genetic ablation of *Pik3cg* leads to reduced tumor growth, angiogenesis, and metastasis in mouse cancer models via downregulation of hypoxic stabilization of hypoxia inducible factor 1α and a decrease of other TAM-related proangiogenic factors, such as VEGF.[130] Furthermore, the lack of PI3Kγ increased the expression of MHC-II, induced the secretion of proinflammatory cytokines (such as IL-12 and IFN-γ), and reduced the secretion of immunosuppressive cytokines, such as IL-10 and arginase. The use of PI3Kγ inhibitors, such as SF1126, has corroborated the results observed in genetic models.[128,130]

In other studies, PI3Kγ pharmacological inhibition/genetic depletion promoted the recruitment and enhanced the cytotoxicity of T cells and led to a reduction in tumor size and a lower number of metastatic events in several cancer models (lung, breast, and head and neck squamous cell carcinoma (HNSCC)). Furthermore, combinatorial inhibition of PI3Kγ and PD1 exhibited additive effects on tumor suppression.[128]

Another recent study tested a PI3Kγ inhibitor in combination with PLG-CA4, a vascular disrupting agent. Treatment with PLG-CA4 in a mammary carcinoma model induced the polarization of TAMs to a protumor phenotype. However, combination treatment of PLG-CA4 and PI3Kγ inhibitor decreased the number of protumoral TAMs and delayed tumor growth.[131] Although PLG-CA4 treatment alone caused a reduction in pulmonary metastasis, the combination of PLG-CA4 and PI3Kγ inhibitor was more effective. These effects might have been partly due to enhanced cytotoxic T cell trafficking into the tumor and to a marked reduction of whole tumor MMP9 expression (MMPs serve as biomarkers of tumor progression and metastatic spread). In the same study, combination of PLG-CA4, PI3Kγ inhibitor, and NLG919 (an inhibitor of immune checkpoint IDO) improved the therapeutic effect of NLG919 alone. In synergy, these agents apparently target tumor vasculature (PLG-CA4) and the immunosuppressive activity of TAMs (PI3Kγ inhibitor) and promote survival and activity of $CD8^+$ T lymphocytes, while suppressing regulatory T cells (NLG919). AZD3458 is a highly selective PI3Kγ kinase inhibitor that remodeled the TME by decreasing the number of TAMs and reducing the overall protein expression of the immunosuppressive markers CD206 and PDL1 in the 4T1 orthotopic breast tumor model.[132] Moreover, AZD3458 reduced MDSC/neutrophil activation and promoted cytotoxic T cell activation *in vivo*. Combination treatment of AZD3458 with checkpoint inhibitor anti-PD1 or anti-PDL1 had greater antitumor effects than checkpoint inhibitor alone in different mouse models.[132]

In terms of therapeutic specificity, PI3Kγ seems to be induced by tumor cell–derived signaling.[133] This suggests that PI3Kγ inhibitors might only affect TAMs, excluding homeostatic macrophages.[134] In the clinic, patients with lung and head and neck cancers and low PI3Kγ activity have better prognosis and longer survival.[128] These data suggest that PI3Kγ could be a potential, specific therapeutic targeting strategy that could be particularly effective in combination with other agents.

Currently, PI3Kγ inhibitors in clinical trials include IPI-549 alone or in combination with AB928 (a dual adenosine receptor antagonist), doxorubicin, or paclitaxel is being tested in triple-negative breast and ovarian cancers.[135] IPI-549 alone or in combination with paclitaxel and/or bevacizumab (anti-VEGF) is also being tested in breast and renal cancer patients;[136] and IPI-549 alone or in combination with nivolumab is being tested in patients with advanced solid tumors, non-small cell lung cancer, melanoma, squamous cell cancer of the head and neck, triple negative breast cancer, bladder cancer, and urothelial carcinoma.[137,138]

*TLR agonists*. TLRs comprise one of the pattern recognition receptor families that control innate immunity.[139] TLR-activating molecular patterns include viral and bacterial nucleic acids,

lipopolysaccharide (LPS), lipoteichoic acid, and mannans (and others) that have been shown to polarize macrophages toward a proinflammatory phenotype. Activation of TLRs via synthetic ligands has been tested in different cancer models with the aim of switching TAMs to a tumoricidal phenotype in the TME. For example, local delivery of TLR7 and TLR8 agonist 3M-052 resulted in tumor regression in a melanoma mouse model, and this effect was due to the change in phenotype of TAMs.[140] Furthermore, a combination of specific TLR agonist with anti-PDL1 and anti-CTLA4 showed a synergistic effect in inhibiting tumor growth; for example, R848, a TLR7 and TLR8 agonist, when delivered to TAMs via β-cyclodextrin nanoparticles in combination with anti–PD1 treatment, led to an increased immune response in various models.[141]

Since September 2015, more than 70 clinical trials have been started to evaluate the therapeutic efficacy of TLR agonists in patients with cancer, including TLR2- and TLR4-stimulating BCG (Bacillus Calmette–Guérin), that is, a live-attenuated *Mycobacterium bovis* enriched in peptidoglycans and unmethylated CG-containing DNA; TLR3 agonists rintatolimod (commercially known as Ampligen®) and poly-ICLC (commercially known as Hiltonol®); TLR4 agonist G100; TLR8 agonist motolimod; TLR9 agonists SD-101, DV281 (Class C CpG-ODN), and DUK-CPG-001 (a synthetic CpG-rich oligonucleotide); and TLR3 and TLR7 agonist imiquimod.

BCG is now the gold standard immunologic agent to treat high-grade nonmuscle-invasive bladder cancer.[142–144] TLR8 agonist imiquimod is being tested in phase III trials and has proven safe as a topical agent for patients with basal cell carcinoma, anal carcinoma, cervical intraepithelial lesions, and other skin carcinomas.[139,145] In preclinical models, lung cancer treatment with aerosolized TLR9 agonist DV281 led to a lung-localized substantial, but transient, cytokine, and chemokine response that was therapeutically beneficial.[146] DV281 is now in a phase 1b/2 study and was proven safe in combination with nivolumab in patients with advanced or metastatic NSCLC.[147]

TLR3 agonist poly-ICLC, TLR4 agonist G-100, TLR8 agonist motolimod, and TLR9 agonist SD-101 have proven safe in clinical trials. G100 used in Merkel cell carcinoma patients increased inflammation in the injected tumors, as indicated by an increase in $CD8^+$ and $CD4^+$ T cells as well as upregulation of immune-related genes; treatment left some patients recurrence-free after 41 months and some with sustained partial responses lasting 33 months.[148] Poly-ICLC generated a local and systemic immune response (i.e., an increase of $CD8^+$ and $CD4^+$ T cells, $CD86^+$ antigen-presenting cells, $CD68^+$ macrophages/monocytes, and $CD16^+$ NK cells at tumor sites) that led to clinical benefit of patients with recurrent metastatic disease (HNSCC or melanoma).[149] In combination with PD1 inhibitor pembrolizumab in patients with unresectable or metastatic melanoma, SD-101 induced immune activation at the tumor sites indicated by increased expression of $CD8^+$ T, NK, dendritic, and B cell–related genes; increased immune activity was variable among patients, but correlated with increased clinical response.[150] Motolimod in combination with cetuximab in patients with recurrent or metastatic HNSCC led to some patients achieving partial responses and some achieving disease stabilization,[151] while in combination with pegylated liposomal doxorubicin in patients with recurrent ovarian cancer, it did not significantly improve overall clinical outcomes. However, subset analyses revealed significant differences of motolimod in patients who showed immune responses *in vitro.*[152] Thus, there is a need to develop strategies to identify and select patients' subsets that might benefit from treatment with this immunomodulatory agent.

**Targeting TAM-preferentially expressed targets/markers.** *LILRB-2*. LILRB-2 belongs to the family of leukocyte immunoglobulin-like receptors (LILR).[153] Inhibitory LILRB receptors are expressed by myeloid cell populations and are primate specific. Paired immunoglobulin-like receptor B (PirB) is the only mouse receptor orthologous to the human LILRB/CD85 family. In murine models, PirB-deficient macrophages show enhanced proinflammatory cytokine release and exacerbate autoimmune disease.[154] In humans, little is known about the role of LILRBs in macrophage activation, in part due to the poor conservation of these receptors between humans and mice; however, because LILRBs bear the immunoreceptor tyrosine–based inhibitory motifs of PirB, LILRBs potentially modulate macrophage behavior as well. This has been borne out experimentally. For example, i*n vitro,*

anti-LILRB-2 treatment increased primary human monocyte–derived macrophage responses to LPS and allowed for the production of inflammatory monocyte–derived macrophages in the presence of CSF1. This response was shown by downregulation of CD14, CD163, and IL-10 and upregulation of TNF-α at the protein level.[155] LILRB2 antagonism suppressed LPS-induced PDL1 (CD274) expression in monocytes and macrophages from human healthy donors. *In vivo*, LILRB2 suppression increased the efficiency of checkpoint inhibitor anti-PDL1 for treatment of Lewis lung carcinoma tumors. While there was a decrease in granulocytic-MDSCs—a tumor immune suppressive myeloid population[101]—and $T_{reg}$ cells in tumors, there was an unexpected increase in monocytic macrophage–derived suppressor cells (M-MDSCs), another myeloid population associated with immune suppressive functions.[101] The accumulation of M-MDSCs did not compromise the efficacy of anti–PDL1 treatment, which suggests that LILRB-2 antagonism is polarizing M-MDSCs to an immunostimulatory/nonimmunosuppressive phenotype, rather than reducing their numbers.

*MARCO*. The macrophage receptor with collagenous structure (MARCO) is a 210 kDa trimeric membrane-bound type II glycoprotein belonging to the class A scavenger receptor family.[156] MARCO expression is restricted to some subsets of macrophages in secondary lymphoid organs[157]; its expression can be upregulated on homeostatic macrophages after bacterial infection or LPS stimulation.[158–160] MARCO expression has also been found in a subset of TAMs in melanoma, breast, colon, and endometrial cancers,[161] and, more recently, NSCLC.[162] Georgoudaki *et al.* explored the potential of repolarizing MARCO-expressing TAMs from a pro- to an antitumoral phenotype as a potential therapy. Anti-MARCO treatment reduced tumor growth and metastatic spread in mammary carcinoma and melanoma murine models. Additionally, in the melanoma and colon cancer models, the combination of anti-MARCO and anti-CTLA4 neutralizing antibodies improved the therapeutic effects of the treatments given on their own. This was explained by an alteration of the composition of TAMs in the TME, as the MARCO-expressing TAM population switched from a tumor promoting to an antitumor phenotype, resulting in the tumor becoming immunogenic and thereby contributing to the reduction of immune suppression within the tumor. MARCO expression was restricted to a subset of TAMs with an immunosuppressive gene signature in mammary, colon, and melanoma carcinoma cell line models.[161] This suggests that anti-MARCO strategies can selectively target antitumor TAMs. Furthermore, a recent study of a large cohort of human NSCLC tumors showed that MARCO is expressed only by a subset of TAMs.[162] Interestingly, $MARCO^+$ cells were found to coexpress PDL1 and have a striking localization pattern: they surround tumor islets/tumor cell nests. This suggests that $MARCO^+$ TAMs could act as a physical and immunosuppressive barrier that protects tumor cells. At the transcript level, MARCO expression correlated with expression of checkpoint molecules PDL1, PD1, CTLA4, and VISTA. These observations suggest that targeting MARCO, in combination with immune checkpoint inhibitors, could be a potential therapy for subsets of NSCLC patients who have a high number of MARCO-expressing TAMs.

### *Function-based reprogramming of TAMs*

**Targeting phagocytic activity of macrophages.** Tumor cells can evade macrophage clearance by overexpressing antiphagocytic surface proteins: this might be a fundamental defense mechanism induced in tumors that enables their survival. The most studied and documented of these antiphagocytic signals is via CD47, which engages with macrophage signal regulatory protein-alpha (SIRP1α).[163,164] Monoclonal antibodies or small molecules that antagonize this interaction have demonstrated therapeutic potential in several cancers; however, there is a strong variability in the efficacy and durability of response and/or relapse, which might be due to redundant inhibitory signals involved in immunoregulation. To date, new antiphagocytic signals have emerged, for example, MHC class I component beta 2-microglobulin ($\beta_2$-M) that binds the macrophage inhibitory receptor LILRB1[165] and CD24 that binds macrophage sialic acid–binding Ig-like lectin (SIGLEC)-10.[166]

**CD47 and SIRP1 axis.** CD47 is a ubiquitously expressed transmembrane protein with one immunoglobulin-like (Ig-like) extracellular domain and five transmembrane domains.[167] CD47 is involved in regulating cellular activities,


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including apoptosis induction, cytokine production, regulation of phagocytosis, cell migration, axon extension, cell–cell fusion, and T cell activation.[168–174] CD47 engages with thrombospondin-1 and SIRP1α. In contrast to CD47, SIRP1α expression is restricted to myeloid cells (monocytes, macrophages, granulocytes, and dendritic cells) and neurons.[167,175] SIRP1α contains three Ig-like extracellular domains and its cytoplasmic domain contains tyrosine-based inhibition motifs (ITIMs) that recruits inhibitory proteins like Src-homology region 2–containing protein phosphatase (SHP) 1 and SHP2.[176,177] In macrophages, binding of SIRP1α to CD47 couples SIRP1α and these tyrosine phosphatases, which ultimately prevents phagocytosis via the functional suppression of nonmuscle myosin IIA at the phagocytic synapse.[176]

In cancer, CD47 is overexpressed in a wide variety of tumor types: from myeloid leukemia and non-Hodgkin's lymphoma (NHL), to solid tumors in bladder and breast cancer.[163,178–185] Several *in vitro* studies have shown that blocking of CD47 with monoclonal antibodies enables macrophage phagocytosis of tumor cells.[163,178,180] Other studies have shown that CD47 abrogation significantly enhances the ability of macrophages to kill tumor cells via antibody-dependent cellular cytotoxicity (ADCC).[13,186–188] *In vivo*, treatment with anti-CD47 reduces tumor burden and improves survival in a wide variety of human tumor–engrafted mice models, thus highlighting that inhibiting CD47 may be a successful strategy for cancer therapy.[164,180,189,190] Currently, several antibodies and fusion proteins that target the CD47–SIRP1α axis are being tested in early phase clinical trials: Hu5F9-G4, CC-90002, SGN-CD47M, IBI188, AO-176, and SRF231 are all CD47 monoclonal antibodies, while CC-95251 and BI-765063 are anti-SIRP1α monoclonal antibodies. Additionally, TTI-621 is a fusion protein containing the sequences encoding the N-terminal CD47 binding domain of human SIRP1α and the Fc domain of human immunoglobulin (IgG1); TTI-622 is similar to TTI-621 but the Fc domain is that of human immunoglobulin IgG4; ALX148 is a fusion protein composed of a modified SIRP1α D1 domain and an inactive human IgG1 Fc; and HX 009 is an anti-PD1–CD47 bispecific antibody fusion protein.

Hu5F9-G4 treatment alone was well-tolerated in clinical trials and led to significant clinical improvement in patients with ovarian and fallopian tube cancer.[191] In combination with azacitidine, it demonstrated objective responses in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) patients,[192] while in combination with rituximab, it showed effectiveness in patients with follicular- and diffuse large B cell–lymphomas.[193] CC-90002 did not show significant results in a trial for patients with AML/MDS[194–196]; however, in combination with rituximab, the need for further clinical evaluation of patients with $CD20^+$ relapsed/refractory NHL was concluded.[197] Initially, TTI-621 raised concerns regarding its safety profile, as patients suffered from moderate to severe thrombocytopenia;[198–200] however, later observations showed that this effect was transient and could be reduced after multiple infusions. TTI-621 intratumor injections caused a measurable improvement in outcomes in cutaneous T cell lymphomas.[201,202] ALX148 alone or in combination with anti-PD1 and anti-HER2 agents proved safe and showed objective responses in patients with late-stage NSCLC, HNSCC, and gastric/gastroesophageal cancer.[203]

Current clinical trial results and the emergence of new agents targeting the CD47–SIRP1α axis underscore the importance of targeting the phagocytic ability of macrophages from the TME. However, there is room for refinement in the targeting strategies against this axis. One of the main concerns is that CD47 is also expressed by nontumor cells. This not only explains why thrombocytopenia and anemia are among the most common treatment-associated side effects but also suggests that anti-CD47 antibodies are likely to be sequestered away from tumor cells by CD47-expressing normal cells. Thus, there is a necessity to monitor and ensure that sufficient levels of anti-CD47 antibody are reached in the blood and TME during treatment. Since SIRP1α is more narrowly expressed than CD47, therapeutic strategies aiming at SIRP1α might result in less toxicity and more effectiveness. However, it is important to keep in mind that these agents may affect solid tissues, such as liver, lung, and brain, which contain large numbers of macrophages.

**$\beta_2$-M of the MHC-I and LILRB1 axis.** Molecules from the MHC class I are composed of a single polymorphic heavy chain and the single light chain $\beta_2$-M.[204] These molecules are known for their antigen-presenting function to cytotoxic T cells and subsequent triggering of adaptive immune

response. Recently, MHC class I has been proposed to act as an antiphagocytic signal.[165] Treatment of 18 different human cancer cell lines with anti-CD47 led to variable magnitudes of macrophage phagocytosis; some cell lines were not phagocytosed at all.[165] These findings suggested the expression of one or more dominant inhibitory signals besides CD47. MHC-I proteins emerged as candidates because there was a putative relationship between high expression of MHC-I proteins and resistance to CD47-induced phagocytosis. Furthermore, human tumor cell lines that lacked both CD47 and MHC-I were the most sensitive to macrophage phagocytosis, when compared with cell lines that expressed at least one or both cell-surface proteins.

LILRB1, a receptor that contains immunoreceptor inhibitory motifs and is involved in intracellular transduction of inhibitory signaling,[205,206] is expressed by macrophages. This receptor binds to MHC-I, which leads to the inhibition of phagocytosis. Barkal *et al.* demonstrated that LILRB1 specifically binds to the $\beta_2$-M subunit of MHC-I, rather than the mostly allele-specific polymorphic heavy chain; abrogation of MHC-I, the $\beta_2$-M subunit, or LILRB1 potentiated phagocytosis of tumor cells both *in vitro* and *in vivo* and led to an additive effect to that of abrogation of CD47. The study by Barkal *et al.* suggested that targeting two independent antiphagocytic axes might be a promising strategy to sensitize tumor cells to attack by macrophages. However, to date, there are no clinical studies aimed at modulating the LILRB1–MHC-I axis.

**CD24–SIGLEC10.** Sialoglycoprotein CD24 has emerged as a novel antiphagocytic signal that binds macrophage SIGLEC10[166] and has a role in adaptive immunity, inflammation, autoimmune diseases, and cancer.[207,208] It is overexpressed in many cancers and appears oncogenic. SIGLEC10 is involved in cell–cell recognition and interaction with sialylated ligands from specific cell populations.[209] At the transcript level, CD24 is very highly expressed in nearly all tumor types,[166] especially in ovarian cancer and triple-negative breast cancer (TNBC) tumors. Interestingly, CD24 expression is higher than that of other well-described antiphagocytic signals (CD47, PD1, and $\beta_2$-M) in most tumor types. Furthermore, stratification of patients by CD24 expression showed a negative correlation between CD24 and overall survival advantage. Single-cell RNA-seq on TNBC samples revealed that CD24 is a tumor-cell specific marker in the TME and that SIGLEC10 is expressed by a substantial fraction of TAMs.[166] Abrogation of CD24 in human tumor cell lines led to enhanced phagocytosis by macrophages; moreover, abrogation of CD24 and blockade of CD47 had a cooperative effect, suggesting that CD47 and CD24 antiphagocytic signals do not serve redundant phagocytic functions. Ablation of SIGLEC10 in macrophages also resulted in an enhanced tumor cell phagocytosis. *In vivo*, injection of CD24-deficient cells led to tumors with reduced size and longer mouse survival compared with injections with wild-type cells; this effect was explained by augmented phagocytosis of infiltrating TAMs.[166] Currently, there is a phase Ib/II clinical trial to test safety and efficacy of combining CD24Fc (a recombinant fusion protein) with ipilimumab and nivolumab in anti-PD1/-PDL1 naive patients who suffer from metastatic melanoma, renal cell carcinoma, or colon cancer.[210]

**Targeting the immunosuppressive activity of macrophages.** TAMs are immunosuppressive because they prevent tumor cell attack by T cells (and NK cells) during tumor progression. In this section, we discuss macrophage programming strategies that lead to the recruitment/enhancement of activated cytotoxic cells.

**PDL1 and PD1 axis.** Programmed cell death protein 1 (PD1) was first discovered as an immune checkpoint receptor that is upregulated on activated T cells to induce immune tolerance.[211–214] When activated, PD1 triggers the phosphorylation of downstream molecules on T cells, namely, CD28, and mitigates the activation of the TCR.[215] Since tumor cells can overexpress the ligand for PD1 (PDL1), they are able to escape the immune system by inhibiting T cell activation. Monoclonal antibodies that target the interaction between PD1 and PDL1 have shown promising clinical efficacy against a wide variety of cancers.[216–222] In the past, PD1- and PDL1-blocking antibodies were assumed to be interchangeable and solely act by interrupting T cell suppression. It is now suggested that anit-PDL1 and anti-PD1 do not function in a completely overlapping manner to promote tumor immunotherapy, and instead anti-PDL1 exerts distinctive, T cell–independent effects on

tumor immunity. Recent studies have revealed that PD1 is not only expressed by T cells but also by B cells, NK cells, dendritic cells, monocytes, and macrophages,[214,223,224] while PDL1 expression has also been shown on TAMs.[214]

The PDL1–PD1 axis in TAMs is difficult to place under one category of TAM reprogramming. We have placed it under "targeting the immunosuppressive activity of macrophages," as targeting PDL1 in TAMs can switch their phenotype to an antitumor one that directly leads to an increase in T cell–mediated immune surveillance.[225,226] However, targeting this axis also modulates macrophage phagocytic ability.[214] *In vitro,* anti-PDL1 treatment of human and mouse macrophages increases macrophage proliferation, survival, and activation, with upregulation of proinflammatory-associated pathways.[227] *In vivo*, anti PD1 therapy was shown to lead to a significant decrease in the number of osteosarcoma lung metastases, enhanced tumor apoptosis, and decreased tumor cell proliferation.[225] Although this anti-PD1 therapy increased NK cell and macrophage tumor infiltration, the numbers of antitumor macrophages were increased, while protumor macrophage numbers were decreased. Macrophage- and NK cell–depletion experiments showed that macrophages were responsible for the effectiveness of the anti-PD1 treatment.[225] Combined PD1–PDL1 antibody treatment in a melanoma murine model also led to tumor regression and enhanced survival compared with single agent–treated and untreated animals. The effects observed were partly explained by a reduced immunosuppressive macrophage phenotype.[227]

Human and murine colorectal TAM PD1 expression directly correlates with the expression of protumor macrophage–associated markers CD206 and CD11c, while it inversely correlates with phagocytic potency against tumor cells.[214] Due to its effect on macrophage, the PD1–PDL1 axis has also been proposed as antiphagocytic axis. *In vivo*, blocking the PD1–PDL1 interaction in a colorectal cancer mouse model, with either anti-PD1 or anti-PDL1 monoclonal antibodies, led to an increase in macrophage phagocytosis, a reduction in tumor growth, and an improved survival rate and duration.[214] Depletion of macrophages reversed the antitumor effectiveness observed with PD1–PDL1 axis blockade. The effects of the PD1–PDL1 blockade on macrophages in human cancer should not be neglected by the focus on T cell signaling, as effects on macrophages may inform therapeutic effectiveness evaluation and suggest other therapeutic approaches.

**SIGLEC1 (CD169).** SIGLEC1, also known as sialoadhesin and CD169, belongs to the sialic acid–binding IgG-like lectin family of proteins.[228] It is expressed by subsets of macrophages in the BM and lymphoid tissues.[226] In humans, SIGLEC1 expression has been associated with shorter disease-specific survival and lower recurrence-free survival in publicly available gene expression data sets from whole tumor homogenates.[229] In breast cancer, SIGLEC1 is one of the top upregulated genes in human TAMs, compared with healthy breast-resident macrophages.[229] Furthermore, SIGLEC1 is upregulated by human macrophages upon their exposure to TNBC cell lines conditioned media.[229]

Depletion of $CD169^+$ macrophages in murine TNBC models has been shown to reduce tumor growth and decreased lung metastasis.[226] Mechanistically, this was explained by a significant expansion of $CD8^+$ T cells in the circulation and spleen, as well as an increased accumulation of these cells within the tumors. *In vitro* culture of $CD169^+$ macrophages with tumor cells caused upregulation of PDL1 in macrophages, which suggests that TAMs in the TME *in vivo* may help subvert T cell–mediated immune surveillance. However, these results may not be an effect of CD169 *per se*, but from depletion of CD169-expressing TAMs.

**MiR-340-5p.** MicroRNAs (miRNAs) are small noncoding RNAs of ∼22 nucleotides in length that silence gene expression in a sequence-specific manner.[230] The maturation of miRNAs is regulated by the RNase-III enzyme DICER. TAMs can be reprogrammed to become tumoricidal macrophages by the modulation of miRNA activity,[231] as conditional deletion of *Dicer* in macrophages leads to hyperactive IFN-γ/STAT1 signaling and recruitment of activated cytotoxic cells into the tumor. Because depletion of DICER results in the loss of TAM immunosuppressive activity and promotes the recruitment of cytotoxic cells, DICER is one of the regulators of the immunosuppressive activity of TAMs.

A recent study proposed that a miR-340-5p–mediated macrophage feedback loop is involved in glioblastoma multiforme (GBM) tumor progression.[232] Low levels of miR-340-5p in GBM

correlate with increased tumor size, recurrence of GBM, and poor survival; and levels of miR-340-5p inversely correlate with protumor TAM density (the TAMs expressed IBA1 and CD163). *In vitro*, overexpression of miR-340-5p in GBM tumor cell lines repressed the recruitment of macrophages and inhibited macrophage activation, resulting in an antitumor state, as shown by a decrease of macrophage expression of CD163, reduced secretion of TGF-β and IL-10, and production of high levels of proinflammatory cytokines, such as IL-6 and TNF-α. *In vivo*, injection of miR-340-5p–overexpressing cells led to lower TAM density in the TME and a lower proportion of protumor TAMs, compared with wild-type control cells; this resulted in a reduction in intracranial tumor volume and prolonged survival. It seems unlikely, however, that this pathway can be targeted therapeutically.

**Macrophage engineering.** An additional provocative therapeutic strategy in preclinical study is the engineering of macrophages to express chimeric antigen receptors (CARs) to kill tumor cells. Several academic laboratories and companies are working on different CAR-expressing macrophage designs to selectively target tumor antigens, such as Her2, and trigger macrophage phagocytosis of cancer cells.[233,234] However, many more studies will be needed to evaluate this therapeutic strategy, as multiple limiting steps, including cell delivery, specificity, survival, effective cancer killing, and toxicity/adverse effects, could potentially prevent CAR-expressing macrophages from moving to the clinic.

## Conclusions and perspectives

The protumor roles of TAMs reported in this review strongly support the idea that targeting macrophages in cancer is a promising therapeutic strategy. All of the preclinical and clinical studies described above suggest that programming macrophages to an antitumor state would be preferential to strategies that deplete all TAMs and/or inhibit their recruitment. Harnessing both macrophage diversity and ability to respond to changing environments can lead to improved therapies, as macrophages with an antitumor phenotype can impair tumor growth via secretion of antitumor factors, phagocytosis, and/or increased immune infiltration, and increased cytotoxicity of T cells and other immune cells. However, there are still several open questions in the field of TAMs that will need careful investigation.

One aspect is the origin of TAMs: different studies have shown that TAMs of different developmental origins accumulate within the TME.[33,38,40] Although both adult BM- and yolk sac/fetal liver embryonic–derived TAMs can be influenced by the presence of the tumor to change their phenotype into protumor cells, they show differences in gene expression profiles.[41] Studies are needed to investigate the interplay of these two populations in tumor progression, invasion, and resistance to therapy, and to answer the following questions: (1) Do TAMs from distinct developmental origins share the same protumor functions? (2) Do they localize in different tumor areas? (3) Can TAMs of embryonic origin proliferate within a tumor? (4) If TAMs of embryonic origin are depleted, could TAMs from adult BM origin replace them, and *vice versa*? (5) Are TAMs of a specific developmental origin more susceptible to depletion and/or reprogramming? To answer these questions, strategies to refine single-cell RNA-sequencing approaches and identify specific markers that distinguish and track TAMs from different origins in mouse and human tumors are needed, like the strategies presented by Zhang *et al.*[42]

A second aspect that requires attention concerns TAM heterogeneity and localization within the tumor area: TAMs can assume different phenotypes depending on their location in a tumor—that is, perivascular, near immune cell–rich or hypoxic areas—however, very little is known about human TAM subset interactions with other immune cells in the presence or absence of therapy. Novel technologies, such as spatial transcriptomics and multiplex immunofluorescence, will likely be pivotal for identifying novel TAM subsets and understanding their function within the TME.

A third aspect requiring attention is the need to compare the TME composition before and after therapy. Are there specific subsets of TAMs that promote therapy resistance? Are these subsets from the same developmental origin? What therapeutic changes are needed to reprogram resistant macrophages to an antitumor phenotype?

Finally, one of the most important aspects will be to investigate the therapeutic clinical efficacy of TAM targeting, mainly in combination with

checkpoint inhibitors, such as anti-PD1 and anti-CTLA4. The challenges for researchers and clinicians will be to find the right targeting strategy, the right timing for the treatment, and the best TAM targets for synergizing with current immunotherapies. Which strategies will be the ones that allow targeting of only the protumor functions of TAMs, while keeping the innate antitumor macrophage properties unaltered? Future studies have a lot to teach us about the fascinating biology of TAMs.

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## Author contributions

M.L.Y., L.C., and J.W.P. wrote the manuscript. J.W.P. conceptualized the manuscript and proofread the final version.

## Competing interests

L.C. and J.W.P. are founders of the company Macomics LTD that aims to target TAM biology in cancer.

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